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Effect of ^{60}Co γ-Irradiation on the Nonspecific Cytotoxicity of Alveolar Macrophages *in Vitro*

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This paper reports on the effect of radiation on the nonspecific cytotoxicity of rat alveolar macrophages (AM). AM (effector cells) of bacille Calmette-Guerin-activated Wistar rats were irradiated with ^{60}Co γ rays *in vitro* to give doses of 0, 100, 300, and 500 Gy. Three hours after irradiation, the AM were cultured with human lung adenocarcinoma AGZY83-a and HeLa target cells in ^{125}I-deoxyuridine-containing media for 6 hr and the cytotoxicity indexes determined. The results indicated that the cytotoxicity indexes of AM against human lung adenocarcinoma cells and HeLa cells were 94.3 ± 0.3% and 81.3 ± 1.9%, respectively. The cytotoxicity indexes using an effector/target cell ratio (E/T) of 10 seemed to be greater than with ratios of 20 and 30. The cytotoxicity indexes of AM (7 rats), irradiated to give doses of 0, 100, 300, and 500 Gy, against adenocarcinoma cells at an E/T ratio of 10 were 87.9 ± 8.4%, 65.4 ± 14.1%, 47.5 ± 17.5%, and 36.7 ± 9.7%, respectively. The significance of the nonspecific cytotoxicity of AM in the immunological elimination of tumors and the inhibitory effect of radiation on AM cytotoxicity are discussed.

Introduction

Alveolar macrophages (AM) are mononuclear phagocytes that reside in the lungs. They are constantly exposed to inhaled pathogenic and/or carcinogenic particulate substances from the environment capable of affecting pulmonary functions.

It has been shown that the growth of lung tumor cells can be inhibited by activated AM both *in vivo* and *in vitro*. Thus, the *in vitro* cytotoxicity index of activated AM might reflect to a certain extent the *in vivo* tumoricidal property of AM and, if tumor-bearing animals were treated with agents toxic to macrophages (silica, carrageenin, etc.), they would rapidly die of pulmonary metastasis (*1–3*).

Evidence suggests that AM play a very important role in host defense against neoplasms and that they constitute an important defense mechanism against particles that reach distal airways. As with other particulate materials deposited in the alveoli, inhaled radioactive particles are readily phagocytized by AM, thus giving a high radiation dose to these cells. AM death has been observed as early as 1 hr after inhalation of $^{239}PuO_2$ particles (*4*). It is expected that of the various cells types present in the lung, AM will be injured most seriously after inhalation of large amounts of radionuclides, and their immunological functions will be suppressed.

However, the inhibitory effect of immunological functions of AM after inhalation of radioactive particles has rarely been studied. As the radiation dose to AM depends on many biological parameters (clearance rate of inhaled particles, changes of renewal rate of AM under different conditions, etc.), the irradiated model of AM for the study of inhaled radioactive particles must be complex. In this study, an *in vitro* model of effects of radiation on AM was used. The aim of this work was to study the inhibitory effect of ^{60}Co γ rays on the nonspecific cytotoxicity of AM and to find protective agents against the inhibition.

Materials and Methods

Wistar rats, 180–250 g, 50–90 days of age, were used in this study. Bacille Calmette-Guerin (BCG) was provided by the Institute of Biological Preparations, Ministry of Public Health, China, was diluted from 75 mg/mL to 15 mg/mL with normal saline solution after heat inactivation at 56°C for 30 min, then stored at 4°C.

RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 20% newborn calf serum was heat inactivated for 30 min at 56°C and supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), and 10 mM Hepes (Sigma, St. Louis, MO). ^{125}I-deoxyuridine (UdR) with a specific activity of 7.4×10^3–1.11×10^4 KBq/mL was used (Academy of Atomic Energy, Beijing, China). Floxuridine (5-fluoro-2-deoxyuridine; FUdR) came from Fluka AG (Chem. Fabrik). Sodium selenite was obtained from Beijing Chemicals Company (Beijing, China). Culture plates had 48 wells of 1 cm² each (Costar Co., Cambridge, MA).

Wistar rats were activated by BCG. Two doses of 3.7 mg/200 g body weight were injected via the caudal vein with an in-

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terval of 7 days. The rats were exsanguinated 2 days after the last injection and their lungs removed and lavaged with 0.02% EDTA in phosphate-buffered saline (PBS) The cells in the pooled lavage fluids from each rat were counted with a hemocytometer in duplicate. Viability was determined by the trypan blue exclusion method. Of the cells recovered, 95% were alveolar macrophages. The AM were suspended in 0.02% EDTA-PBS at a concentration of 10^6/mL in a 30-mL flask.

The flask containing the suspension of AM harvested from BCG-activated Wistar rats was irradiated at a distance of 0.5 m from a gamma source of 2.6×10^{15} Bq ^{60}Co, giving a dose rate of 54–58 Gy/min at 15–25°C. In this experiment, the AM were divided into four groups, receiving absorbed doses of 0, 100, 300, and 500 Gy, repectively.

After irradiation *in vitro*, the suspensions of AM (effector cells, E) were centrifuged at 1000 rpm for 5 min, then the supernatants were removed and the cells were resuspended at different concentrations in RPMI-1640 complete medium. After preculture at 37°C in 5% CO_2 for either 3 hr or 26 hr, a given number of AM was added to each well of the culture plate, together with human lung adenocarcinoma AGZY83-a cells or HeLa cells (target density: 500 cells/mm²) according to a fixed E/T (effector/target) ratio of 10, 20, or 30. Samples were incubated under the same conditions for 22 hr and labeled with ^{125}I-UdR (7.4×10^3 Bq/sample) for 6 hr. The supernatants were then aspirated, and the adherent cells were washed twice with Hank's balanced salt solution. The remaining adherent tumor cells and AM were removed and the ^{125}I-UdR activity incorporated into target cells was determined with a γ counter. A target cell sample processed in the same way but without AM served as the control. The results were expressed as the cytotoxicity index (CI%) calculated according to the following equation:

$$CI\% = \frac{A-B}{A} \times 100$$

where A is the ^{125}I activity of target cells and B is the ^{125}I activity of target cells incubated with AM.

Results

Experimental Model of AM *in Vitro*

To determine the nonspecific cytotoxicity of AM *in vitro*, several preliminary experiments were carried out.

Selection of the Target Cell. A human gastric carcinoma tumor cell line, a Hela cell line, and a human lung adenocarcinoma cell line, AGZY83-a, were labeled with ^{125}I-UdR for 6 hr; the percentages of ^{125}I-UdR incorportated in these tumor cells were 1.9, 3.7, and 5.3%, respectively. The human lung adenocarcinoma cell line had the highest incorporated activity of the three cell lines tested.

Selection of Target Density. Human lung adenocarcinoma cells at densities of 250, 500, 750, and 1000 cells/mm² were labeled with ^{125}I-UdR for 6 hr. The resulting specific activities were 0.051, 0.058, 0.050, and 0.051 cpm/cell, respectively. No significant difference was found within the range of densities tested. The target density of 500 cells/mm² was selected to determine CI% because it mininized the number of tumor cells required while the total activity per sample was high enough to ensure the accurate measurement of incorportation.

Effect of E/T Ratio. The close contact required between effector and target cells could be achieved either by increasing the number of both types of cell or by raising the E/T ratio. The nonspecific cytotoxicity of BCG-activated AM was determined at E/T ratios of 0.1, 1.0, 5.0, 10, 20, and 50. The changes of CI% after 3 hr preculture are shown in Figure 1. The CI% of BCG-activated AM increased with increasing E/T ratio in the range of 0.1%20 and with E/T ratios in the range 20–50. All CI values exceeded 90%.

Selection of Preculture Time. The nonspecific cytotoxicity of BCG-activated AM could be influenced by preculture time *in vitro*. The values in Table 1 show that the CI% decreased significantly at all E/T ratios tested when preculture times were prolonged from 3 hr to 26 hr. With an E/T ratio of 0.1, AM were no longer cytotoxic to the tumor cells after 26 hr of preculture. This effect could be attributed to a decrease of active effectors for killing tumor cells after prolonged preculture time.

Taking all these results into consideration, we chose the human lung adenocarcinoma AGZY83-a and the HeLa cell lines as the target cells, (target density 500 cells/mm² and precultured for 3 hr) for the *in vitro* model used in the following experiment.

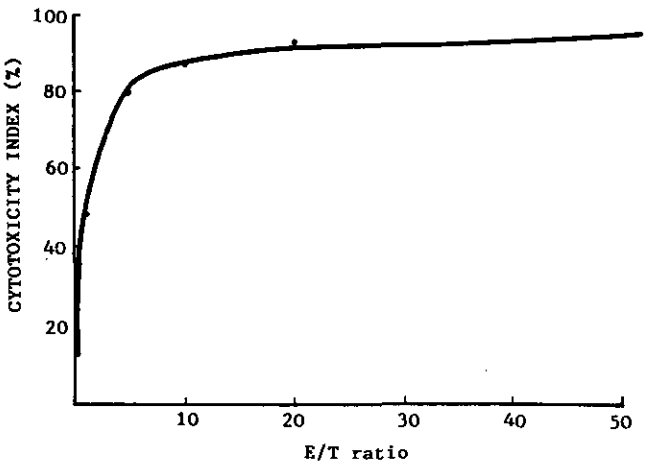

FIGURE 1. Cytotoxicity index of bacille Calmette-Guerin-activated alveolar macrophages at different effector/target (E/T) ratios.

Table 1. The cytotoxicity index of BCG-activated alveolar macrophages after 3 or 26 hr of preculture.[a]

	Cytotoxicity index of BCG-activated AM, %	
E/T ratio	3-hr preculture	26-hr preculture
0.1	22.0 ± 8.0	-0.8 ± 10.7*
1.0	74.9 ± 0.8	34.2 ± 12.6†
2.5	82.4 ± 2.8	62.4 ± 15.2*
5.0	85.0 ± 2.4	59.0 ± 10.5†
20.0	91.0 ± 0.9	67.5 ± 6.0
50.0	93.9 ± 4.0	77.1 ± 4.5†

Abbreviations: BCG, bacille Calmette-Guerin; E/T, effector/target; AM, alveolar macrophage.

[a]Values are means ± SD, $n = 6$.

*Statistically significant difference as compared with the cytotoxicity index of 3-hr preculture group, $p < 0.01$.

†Statistically significant difference as compared with the cytotoxicity index of the 3-hr preculture group, $p < 0.001$.

Table 2. Effect of ^{60}Co γ rays on the cytotoxicity index of BCG-activated alveolar macrophages against two tumor cell lines (means ± SD).

Dose, Gy	Cytotoxicity index	
	HeLa cell line	Adenocarcinoma cell line
0	81.3 ± 1.9	94.3 ± 0.3
100	75.5 ± 1.0	92.9 ± 0.9
300	59.0 ± 1.6	80.0 ± 0.1
500	50.9 ± 7.2	60.7 ± 2.6

BCG, bacille Calmette-Guerin.

Effect of ^{60}Co γ Rays on Nonspecific Cytotoxicity of BCG-Activated AM *in Vitro*

AM, activated by BCG, were very effective in killing both AGZY83-a cells and HeLa cells. The results of cytotoxicity determinations with doses of 100–500 Gy, using irradiated AM cultured at an E/T ratio of 20 after 3 hr preculturing (Table 2), indicate that the CI of irradiated AM decreased with increasing radiation dose. The inhibition was more pronounced in adenocarcinoma cells than in HeLa cells.

Additional studies of the inhibitory effect of radiation on the CI% of BCG-activated AM against the AGZY83-a cell line were made with E/T ratios of 10, 20, and 30. The results are listed in Table 3, showing a more severe effect at an E/T ratio of 10 than at 20 and 30.

To confirm the relationship between radiation and inhibition of CI% of BCG-activated AM, repeated determinations of the dose-response relationship were made on seven rats at an E/T ratio of 10, using AGZY83-a cells as the target. The results shown in Figure 2 were obtained. The CI% of AM was about 90% in the control group, and consistently decreased as the radiation dose increased from 100 to 500 Gy. With a dose of 500 Gy, a CI of only 42% of the normal value was found. Thus, it is beyond doubt that the radiation-induced inhibition of CI% is dose dependent.

Table 3. Effect of ^{60}Co γ rays on the cytotoxicity index of BCG-activated alveolar macrophages at different E/T ratios (means ± SD).

Dose, Gy	Cytotoxicity index, %, at E/T ratios of		
	30	20	10
0	94.2 ± 0.6	94.3 ± 0.3	94.0 ± 0.3
100	92.1 ± 0.7	92.9 ± 0.9	88.1 ± 2.6
300	84.3 ± 0.8	80.0 ± 0.1	71.8 ± 3.7
500	63.4 ± 4.0	60.7 ± 2.6	44.9 ± 1.2

Abbreviations: BCG, bacille Calmette-Guerin; E/T, effector/target.

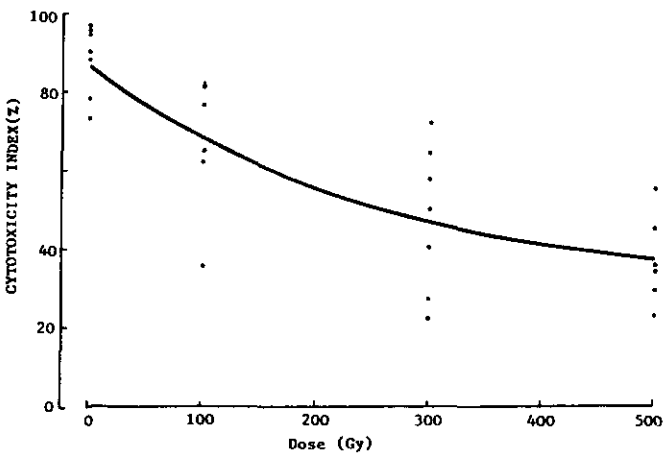

FIGURE 2. Effect of different doses of radiation on cytotoxicity index of bacille Calmette-Guerin-activated alveolar macrophages.

Possible Protective Effect of Selenium on Radiation-Induced Inhibition of Cytotoxicity Index

The manifestation of cytotoxicity of AM depends on cell membrane integrity. It is well known that selenium, as an important component of glutathione peroxidase (GSH-px), can promote the primary function of GSH-px *in vivo*. It has also been shown that the unsaturated fatty acid or lipid components of cell membranes form peroxides when irradiated in an aqueous system (*5*). Thus. Thus, the addition of selenium to irradiated AM should protect the cell membrane from peroxidation, thereby maintaining its integrity. To investigate this hypothesis, we carried out the following experiment.

One hour before irradiation, selenium (sodium selenite) at a concentration of 10 μM was added to the control and irradiated AM cultures. Two hours after irradiation, the selenium was removed by aspiration and washing with Hank's balanced salt solution. Then the AM were suspended in RPMI-1640 complete medium, and the CI% was determined. From Table 4 it can be seen that selenium has a protective effect on the inhibition of CI% of irradiated AM as the CI increased from 14.8 to 43.3%. It appears that there is a definite correlation between cytotoxicity and integrity of membranes, and the intact membrane might be quite an important effector for the manifestation of nonspecific cytotoxicity of AM.

Discussion

AM are mononuclear phagocytes that locate anatomically at the interface between tissue and air. This kind of phagocyte forms the first line of defense in the respiratory system. AM inactivate toxic particles and play an important role in the anticancer process. The results described indicate that BCG-activated AM can kill HeLa cells and human lung adenocarcinoma cells. We also observed tumoricidal ability of AM, activated by BCG, when melanoma B-16 cells were used as a target. These results show that activated AM have the ability to kill or inhibit various kinds of tumor cells. It is an essential function of AM to defend the organism against tumors. The inhibition of CI% was dependent on radiation dose to AM and on the E/T ratio. To illustrate the mechanism of radiation effect, the viability of AM was determined by the trypan blue exclusion method. This showed that 87% of the AM were viable in the control and 84–89% in the three irradiated groups. There was no difference in mortality of AM after irradiation. It seems that the inhibitory effect on CI was not caused by direct killing. It has been shown that cytoplasmic and lysosomal membranes of AM were damaged following irradiation with 100–500 Gy γ rays (*6*), which might explain the decrease of CI% in irradiated AM.

Table 4. Protective effect of selenium against radiation-induced inhibition of cytotoxicity index of irradiated alveolar macrophages (mean ± SD).

Sodium selenite, mole/mL	Cytotoxicity index, %	
	Control AM	Irradiated AM (300 Gy)
0	67.9 ± 1.3	14.8 ± 17.4
1×10^{-5}	54.2 ± 4.9	43.3 ± 6.9

AM, alveolar macrophages.

The preliminary results of the observation of a protective effect of selenium on radiation-induced inhibition of CI% at a dose of 300 Gy were of significance in illustrating the dependence of AM cytotoxicity on cell membrane integrity. It will be necessary to carry out further experiments to confirm this observation.

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